Increased Expression of the M, 72,000 Type IV Collagenase in Human Colonic Adenocarcinoma


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ABSTRACT

Proteolytic enzymes, such as type IV collagenase, play an important role in tumor invasion and metastasis. To examine M, 72,000 type IV collagenase expression in human colon carcinoma, blot hybridizations of total RNA from 19 primary colon tumors were performed. These filters were probed with complementary DNA probes encoding the M, 72,000 type IV collagenase metalloenzyme. The results were expressed as the ratio of the messenger RNA (mRNA) levels in the tumor tissue to that in the adjacent normal mucosa (R). The level of the 3.1-kilobase type IV collagenase mRNA was higher in the primary tumor than in the normal adjacent colon mucosa in 13 of 18 (72%) cases with a diagnosis of adenocarcinoma. These cases were divided into high expression (R, 4.50 to 29.34) and intermediate expression (R, 2.54 to 3.31) subgroups. Both groups showed statistically significant (P < 0.05) elevations when compared with the five cases showing the lowest levels of M, 72,000 type IV collagenase mRNA expression (low expression subgroup; R, 0.96 to 1.48). With this demonstrated elevation of M, 72,000 type IV collagenase mRNA in colorectal adenocarcinoma we examined concomitant expression at the protein level using immunohistochemical techniques. Immunohistochemical examination of 70 cases of colon tumors, including 30 benign adenomas, using anti-M, 72,000 type IV collagenase antibodies demonstrated a significant correlation with Duke's classification (P < 0.001). Our results suggest that enhanced expression of the M, 72,000 type IV collagenase enzyme may be a marker of human colorectal tumor invasiveness.

INTRODUCTION

Colorectal carcinoma ranks second to lung cancer as the leading cause of cancer deaths in the adult United States population (1). It has been estimated that, of the 151,000 new cases of colorectal cancer diagnosed in 1989, over 40% will succumb to the disease. Local invasion and distant metastases are the primary causes of treatment failure in patients with colorectal cancer (2). Recent animal studies have clearly demonstrated that tumor cell invasion and metastasis are not random events but arise from a tumor cell subpopulation of higher metastatic potential (3–5). For colorectal neoplasia, tumor size and therefore local tumor burden may bear little relationship to invasive potential (6, 7). Thus, recent work has focused on biochemical and genetic markers of the invasive phenotype, which would allow identification of patients at greater risk for subsequent development of metastatic disease.

Studies demonstrated both ras gene mutations and myc gene amplification in colorectal carcinomas (8, 9). However, these studies failed to demonstrate a correlation between the degree of tumor invasiveness and the presence of mutated oncogenes. Recent cytogenetic and molecular studies showed allelic deletion of chromosomes 5q, 18q, and 17p, as well as ras mutations in colorectal cancer (10). Although these studies correlated mutations and chromosomal changes with the stage of the tumor, particularly progression of adenoma to carcinoma, data on a much larger population of tumors are required to define which of these properties is independently correlated with the specific genetic alterations seen in these tumors.

The basement membrane is a continuous extracellular matrix containing laminin, type IV collagen, heparan sulfate proteoglycan, and other minor macromolecular components. It functions to separate organ parenchyma from underlying stroma. Loss of basement membrane in colorectal adenocarcinoma and in gastric carcinoma is indicative of an increased probability of distant metastasis and a poor prognosis (11–13). Augmented proteolysis of basement membrane collagen is a potential mechanism for the disruption of basement membranes that is associated with tumor progression and invasion.

The M, 72,000 type IV collagenase is a neutral metalloproteinase capable of degrading basement membrane (type IV) collagen within the triple helical domain to yield characteristic one-fourth amino terminal and three-fourths carboxy terminal fragments (14, 15). This enzyme has been closely associated with the metastatic phenotype. Studies with transfected cell lines have shown a linkage between augmented M, 72,000 type IV collagenase activity and the genetic induction of the invasive phenotype (16–18). A study of both the interstitial and the type IV collagenolytic activity associated with colorectal carcinoma tissues showed that these activities were elevated in 4 of 29 malignant primary tumors (19). All other primary tumors secreted only slightly higher levels of collagenolytic activities. Selected human colorectal tumor cell lines derived from human colorectal carcinoma tissues have shown a correlation between type IV collagenase activity and the frequency of in vivo experimental metastasis in nude mice (20).

We have observed that highly invasive tumor cell lines secrete both the M, 72,000 type IV collagenolytic activity and specific inhibitors of this enzyme (21). This suggests that this enzyme activity is closely regulated and that direct measurement of the type IV collagenolytic activity following trypsin activation might not reflect the in vivo collagenolytic potential. To develop a better understanding of the relationship between M, 72,000 collagenase activity and the invasiveness of colorectal tumors we examined the levels of M, 72,000 type IV collagenase mRNA transcripts in 18 matched pairs of colon adenocarcinoma tissue and adjacent normal mucosa and one colonic adenoma and adjacent normal tissue. The results demonstrate that 72% (13 of 18) of all malignant tumors contained significantly elevated type IV collagenase transcript levels. Furthermore, immunohistochemical studies demonstrate that the augmented steady state levels of M, 72,000 type IV collagenase mRNA result in increased synthesis of type IV collagenase enzyme by the tumor cells. This increased enzyme synthesis correlated with the histopathological staging of the colorectal tumor.
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MATERIALS AND METHODS

Isolation and Sequence Analysis of Full Length cDNA Clones for Human M, 72,000 Type IV Collagenase. Plaques (2 × 10^6) of a Agt11 human placenta cDNA library (Clontech) were screened with a 100-mer oligonucleotide probe derived from the 100 base pairs of the cDNA sequence of the M, 72,000 type IV collagenase as reported by Collier et al. (22). This probe, referred to as P100–5, was synthesized on a Biosearch 8700 DNA synthesizer. P100–5 was labeled using [γ-32P]-ATP (6000 Ci/mmol, ICN Radiochemicals) and T4 kinase (BRL). Positive plaques were purified, subcloned into pUC18, and sequenced using the dideoxy chain termination method (23). A single 1.1-kilobase clone, pH3a, was selected and used to rescreen the Agt11 human placental cDNA library. This resulted in identification of a single 1.1-kilobase clone, which was subsequently purified, subcloned into pSPT19 (Pharmacia), and sequenced.

Primer Extension Characterization of M, 72,000 Type IV Collagenase Clones. Oligonucleotide primers 74 (5'-CTCAGCGGCTCATGG-3') and 75 (5'-CATGGTCCGGCCCCCGCCCCCA-G-3') were synthesized. Primer extension experiments were performed according to the procedure described by Rao et al. (24) using mRNA from human A2058 melanoma cells and Superscript MMLV-reverse transcriptase (BRL). mRNA was isolated from A2058 cells using the Fast Track kit (Invitrogen).

RNA Extraction from Human Tissue Samples. Human colon tumor tissue and adjacent normal colon mucosa samples were obtained from partial colectomy surgical specimens. Nineteen colorectal tumors were examined. These included one benign adenoma and 18 colorectal cancers. Review of the histopathology reports for these 18 cases of malignant colorectal tumors revealed that there were 9 Duke's stage B tumors (3 B1 and 6 B2), 6 Duke's stage C, and 3 Duke's stage D tumors. All malignant tumors were classified as adenocarcinomas with or without mucin production. Tissue was immediately frozen in liquid nitrogen and stored at −70° until processing. Frozen tissue was pulverized, and total cytoplasmic RNA was prepared by guanidine isothiocyanate extraction and centrifugation through CsCl (25).

Northern Blot Analysis. Five μg of each total RNA were size-fractionated through 1% w/v agarose gels containing 2.0 M formaldehyde (26). RNA was transferred to GeneScreen Plus membranes (DuPont) by capillary transfer in 10× SSC overnight. The membranes were washed in 2× SSC and UV-cross-linked with 120,000 μJ/cm2 using a UV Stratalinker 2400 (Stratagene). After prehybridization, the membranes were hybridized overnight at 42°. cDNA probes were radiolabeled with [32P]dCTP by the random primer method. Hybridized filters were washed in 0.2x SSC/1% sodium dodecyl sulfate at 65° for 30 min before autoradiography at −70° with intensifying screens. Signals were quantitated using scanning laser densitometry. Slot blots were done using a Bio-Rad microfiltration apparatus. Four dilutions of each total RNA were blotted to Zeta-Probe membranes (Bio-Rad), UV cross-linked, and treated as described for Northern blots.

Immunoperoxidase Staining. Seventy human colorectal tumors obtained at the time of primary resection for diagnosis and treatment at the Istituto di Anatomia Patologica, University di Bologna, were studied using immunohistochemical techniques. The immunoperoxidase staining of 30 cases of colon adenoma was compared with that of 30 cases of Duke's A or B stage carcinoma and 10 cases of Duke's C stage carcinoma. Staining was quantitated by determining the percentage of cells positive in the primary tumors. Immunoperoxidase staining was done using the method of Hsu et al. (27) and the Avidin-Biotin system (PK4002; Vector Laboratories). Rabbit anti-type IV collagenase antibody antibodies were prepared, affinity purified, and verified by immunoblotting and antigen competition of immunohistochemical staining (28, 29). Type IV collagenase immunoreactivity was cytoplasmic. In sections of heterogeneous immunoreactivity, areas with both high and low percentages were considered and the representative fields were tabulated. The scores were calculated as the number of cells positively stained at primary antibody dilutions greater than 1/200 and were divided by the total number of neoplastic cells. In each specimen a minimum of 1000 neoplastic cells was analyzed by three observers. Student's t test was used for comparison of mean values.

RESULTS

Isolation and Characterization of Full Length cDNA Clones for Human M, 72,000 Type IV Collagenase. Screening of a human placental cDNA Agt11 library was performed using the oligonucleotide probe P100–5. This resulted in identification of a 1.1-kilobase cDNA clone, pH3a, which contained 324 nucleotides upstream of the Mr 72,000 type IV collagenase cDNA sequence reported by Collier et al. (22). Clone pH3a was used to rescreen the cDNA library to obtain larger clones. This resulted in identification of a single 3.1-kilobase cDNA sequence reported by Collier et al. (22). Clone pH3a was used to rescreen the cDNA library to obtain larger clones. This resulted in identification of a single 3.1-kilobase cDNA clone, pIV-16. Restriction mapping and sequencing of this clone revealed that it contained all of the 5′ sequence present in clone pH3a in addition to the entire sequence reported for the 3′ end of the M, 72,000 type IV collagenase cDNA sequence reported by Collier et al. (22). The 5′ sequences of both clones are shown in Fig. 1. This sequence contains a 286-nucleotide 5′ untranslated region and encodes for a 29-amino acid signal peptide sequence for M, 72,000 type IV collagenase.

Primer extension reactions were performed using two oligonucleotide primers complementary to sequences in the 5′ end of clone pIV-16 shown in Fig. 1. Primer extension products of 144 and 134 base pairs were obtained using primers 74 and 75, respectively (Fig. 2). These results demonstrate that there is a single transcriptional start site for M, 72,000 type IV collagenase in A2058 melanoma cells and that this site is located 50 base pairs upstream of the end of the pH3a and pIV-16 clones.
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**Fig. 3.** Northern blot analysis of total cellular RNA from colon tumor tissue and adjacent normal mucosa. Five μg of each sample was electrophoresed and transferred to GeneScreen Plus membranes as described in “Materials and Methods.” The diagnosis for patients 1, 3, and 4 was adenocarcinoma of the colon and that for patient 2 was tubulovillous adenoma (see Table 1). Hybridization with α[32P]dCTP-labeled M, 72,000 type IV collagenase cDNA resulted in a single band at 3.2 kilobases. α[32P]dCTP-labeled β-actin cDNA was used to normalize sample loading. The ratio of type IV collagenase expression to expression of β-actin in normal tissue is shown below each lane.

**Table 1: Demographic data, diagnosis, stage of malignancy, and R value**

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<tr>
<th>Patient</th>
<th>Age/Sex</th>
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<th>R value</th>
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® Ratio of tumor type IV collagenase to adjacent normal tissue type IV collagenase levels. Levels of type IV collagenase in both normal and tumor tissues were normalized to human β-actin levels to account for variation in mRNA loading.

Adeno, adenocarcinoma; MD, moderately differentiated; NA, not applicable; WD, well differentiated; PD, poorly differentiated; (N), necrosis of primary tumor noted on histopathological examination.

**Fig. 2.** Transcriptional start site of the human type IV collagenase gene in A2058 melanoma cells. α[32P]ATP-labeled oligonucleotides 74 and 75 (see Fig. 1) were each annealed to 2 μg mRNA from A2058 cells and extended using reverse transcriptase. Primer extension products were electrophoresed through a 10% acrylamide/urea gel. α[32P]ATP-labeled molecular weight markers were used to determine size. Arrows, single primer extension products of 144 and 134 bases for primers 74 and 75, respectively.

**M, 72,000 Type IV Collagenase mRNA Levels in Human Colorectal Tumors Relative to mRNA Levels in Adjacent Normal Mucosa.** Total cellular RNA from 19 matched pairs of human colorectal tumor and normal colonic mucosa were examined for expression of M, 72,000 type IV collagenase mRNA by Northern and/or slot blot analysis. Northern blot hybridization of the initial four cases (Fig. 3) revealed a single band at 3.1 kilobases. Normalization of the data to actin revealed that, in 3 of the 4 cases shown, there was a 4.7- to 7-fold increase in the steady state level of M, 72,000 type IV collagenase mRNA in the colorectal tumors compared with the control tissue. The numbers of the samples in Fig. 3 correspond to the patient numbers in Table 1. Examination of the final diagnosis in these four cases revealed that sample/patient number 2, which had the lowest level of type IV mRNA detected in this study, was a tubulovillous adenoma. High levels of type IV collagenase mRNA were found in those patients (patients 1, 3, and 4) with invasive carcinomas.

All samples were screened for steady state mRNA levels of M, 72,000 type IV collagenase by slot blot hybridization of total cytoplasmic RNA samples. These blots were also hybridized utilizing a human β-actin probe for normalization. Autoradiographs were scanned using a laser densitometer for mRNA quantitation. These results were then expressed as a ratio of M, 72,000 type IV collagenase mRNA in the tumor tissue to the type IV collagenase in the patient matched normal tissue (R value). Thus, a steady state level of M, 72,000 type IV collagenase that was near that of the adjacent normal tissue would result in an R value close to unity. An elevated R value reflects
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Fig. 4. Immunohistochemistry of M, 72,000 type IV collagenase in human colorectal mucosa and tumor tissues. A, normal colonic mucosa showing lack of immunoreactivity in the colonic epithelial cells. Scattered positive histiocytes serve as an internal control for the antibodies. B, invasive adenocarcinoma of the colon (Case 18). There is strong, cytoplasmic immunostaining of the neoplastic colonic epithelia.

elevation of the M, 72,000 mRNA transcript relative to normal tissue mRNA levels.

M, 72,000 type IV collagenase expression was significantly increased in tumor tissue over normal adjacent tissue in 13 of 18 (72%) cases with a diagnosis of invasive colorectal carcinoma (Table 1). The mean R of all invasive colorectal tumor tissues examined was 7.46. The R values showed three distinct groups. Nine of the 18 cases revealed significant elevation of the R value, with values in the range of 4.50 to 29. The average R value for this high expression group was 12.96. The group
expressing intermediate levels of \(M, 72,000\) type IV collagenase mRNA contained \(R\) values ranging from 2.54 to 3.31, and the average value in this group was 2.96. The low expression group contained cases with \(R\) values ranging from 0.96 to 1.48, with an average of 1.15. Comparison of these three groups using an unpaired Student's t test revealed that the high and intermediate \(M, 72,000\) type IV collagenase mRNA expression groups were statistically different in terms of their \(R\) values from the low expression group, \(P < 0.01\) and \(P < 0.0005\), respectively. Comparison of the \(R\) values for the high and intermediate expression groups reveals that they are statistically significant at the \(P < 0.05\) level.

No correlation of mRNA levels and Duke's stages was observed. There was excellent correlation between the Northern blot and slot blot results. Three of four patients (patients 1–3) showed similar levels of elevated \(M, 72,000\) type IV collagenase transcripts when measured by Northern blot hybridization (Fig. 3) or when determined by slot blot hybridization (Table 1). In the single case of a patient diagnosed with a tubulovillous adenoma (patient 2), the \(R\) value was the lowest detected in this study (\(R = 0.49\); Table 1) and allowed easy identification of this sample.

Immunohistochemical Staining of Human Colorectal Tumors and Normal Mucosa. Immunohistochemical staining of invasive colorectal tumor tissue and adjacent normal tissue confirmed the elevated levels of type IV collagenase associated with the tumor samples as seen by analysis of mRNA (Fig. 4). Type IV collagenase immunoreactivity was cytoplasmic as previously reported (29). There was a significant increase in positive cells (\(P < 0.001\)) when comparing percentages of immunoreactive cells present in adenomas [mean, \(8.2 \pm 10.5\) (SD)] and Duke's stage A/B carcinomas [mean, \(30 \pm 15.5\)] (Fig. 5). Furthermore, tumor progression, as measured by comparison of tumors from Duke's stage A/B with Duke's stage C, was also correlated with a statistically significant increase in the percentage of cells staining positive (Fig. 5).

DISCUSSION

Two cDNA clones for \(M, 72,000\) type IV collagenase were isolated, sequenced, and characterized. These clones contained 5' nucleotide sequences not previously reported for \(M, 72,000\) type IV collagenase cDNA clones. The 3' regions of these clones contained sequences identical to that previously reported for type IV collagenase cDNA (22), which confirmed their identity. Primer extension experiments demonstrated that both clones are 50 base pairs short of the transcription start site in human A2058 melanoma cells. The transcriptional start site occurs 284 base pairs upstream of the translational start site. This suggests that the 3.1-kilobase pIV-16 clone is nearly full length. Both Huhtala et al. (30) and Collier et al. (22) were unable to isolate full length cDNA clones for \(M, 72,000\) type IV collagenase and attributed this to complex secondary structure in the 5' end of the gene. Examination of the sequence for this region of the \(M, 72,000\) type IV collagenase cDNA (Fig. 1) reveals several sequence domains of extremely high GC content. These regions may be responsible for the difficulty in obtaining a full length cDNA clone. The 5' ends of these clones are identical to the sequences obtained from human genomic clones of type IV collagenase characterized by Huhtala et al. (30). Furthermore, the results of primer extension experiments are in excellent agreement with one of the transcriptional start sites in human HT1080 fibrosarcoma cells as reported by Huhtala et al. (30). Differences in transcriptional initiation sites may be accounted for by differences in the types of cells used for the RNA template and differences in the nature of the RNA template [total cytoplasmic RNA versus oligo(dT)-selected mRNA].

Analysis of RNA from human colorectal tissues (Fig. 3; Table 1) showed that, in 13 of 18 cases (72%) of invasive colorectal carcinoma, there was a statistically significant increase in the steady state mRNA levels in the tumor tissue compared with adjacent normal tissue. These increases ranged from 2.54- to 3.31-fold for the intermediate expression group (\(P < 0.0005\)) and 4.50- to 29.34-fold for the high expression group (\(P < 0.01\)). A low expression group consisting of five cases of colorectal carcinoma, there was a statistically significant increase in the percentage of cells staining positive (Fig. 5).
membrane. A single case of tubulovillous adenoma (benign polyph, Patient 2, Table 1) was remarkable for the extremely low levels of type IV collagenase mRNA present (R = 0.49). The lack of correlation between the mRNA levels for the M, 72,000 type IV collagenase and the Duke’s levels of the tumor studied, despite the correlation seen at the immunohistochemical level, is probably due to the difficulty in processing human tumor tissue samples for analysis of mRNA.

Finally, immunohistochemical studies showed that the elevated M, 72,000 type IV collagenase mRNA levels in the tumor tissue correspond with increased malignant tumor cell-associated type IV collagenase enzyme. This is an important point because it definitively demonstrates that the overproduction of the enzyme, detectable in tumor tissue samples by hybridization analysis of mRNA levels, is localized principally to the invasive tumor cells themselves and is not due to type IV collagenase production by interstitial stromal cells, fibroblasts, or tumor-associated macrophages. Quantification of the number of tumor cells staining positive for M, 72,000 type IV collagenase showed a definite correlation with the stage of the colorectal tumor that was statistically significant (P < 0.001). Examination of a larger patient population will allow confirmation of this correlation.

This study reports the first evidence that levels of type IV collagenase mRNA transcripts are increased in invasive human colon adenocarcinoma tissue and that this increase is specifically associated with increased synthesis of M, 72,000 type IV collagenase protein in the invading tumor cells. Previous studies using metastatic cell lines demonstrated similar results (17, 31–33). Our results indicate that elevation of M, 72,000 type IV collagenase may be a useful marker for invasive adenocarcinoma in human colorectal tumor samples.

ACKNOWLEDGMENTS

The authors would like to thank Mary Wacher and Inger M. Margulis for technical assistance in antibody purification and immunoperoxidase staining and Edward Unsworth for synthesis of oligonucleotides.

Portions of this work were performed by A. T. Levy in partial fulfillment of requirements for a Master of Science at Hood College, Frederick, MD.

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